

treat diabetes mellitus,” (2) “[t]he specification does not teach that the transplanted pseudo-islet like aggregates act like healthy islet cells,” and (3) “[s]ince claim 25 recites that the patient does not serve as the donor for the nestin-positive pancreatic stem cells, the skilled artisan cannot predict that the differentiated pseudo-islet like aggregates can be successfully immunologically transplanted into the recipient patient.”

Applicants assert that in view of Applicants’ response to the December 4, 2002 Office Action, filed on April 4, 2003, claims 25-35 and 67-79 are fully enabled. The reference cited below provides additional support for enablement of claims 25-35 and 67-79 of the instant application.

It is well established that post-filing documentation or publications may be used to demonstrate that Applicants disclosure was enabling at the time of filing (*See, e.g., In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995)). Accordingly, Applicants submit herewith, Exhibit A which demonstrates that the methods disclosed by Applicants in the present application were sufficient to enable one of skill in the art to practice the claimed method of treating a patient with diabetes mellitus as of the filing date.

Applicants submit that there is post-filing date literature teaching treatment of diabetic NOD-SCID mice by implantation of islet-like cell clusters (ICCs) derived from cultured human fetal nestin-positive islet-derived progenitor cells. (See Exhibit A, Huang and Tang, “Phenotype Determination and Characterization of Nestin-positive Precursors Derived from Human Fetal Pancreas,” *Laboratory Investigation*, 83(4): 539-547 (2003)).

This publication demonstrates the actual reduction to practice of the invention of the instant application, as claimed in independent claims 25 and 67. “Pseudo-islet like aggregates” are defined in the application at page 13, lines 17-21. Applicants submit that one of skill in the art would accept that the nestin positive ICCs described in Exhibit A are equivalent to the “pseudo-islet like” aggregates of the instant application. That is, the nestin positive ICCs of Exhibit A are “artificial aggregates of insulin-secreting cells which resemble in form and function the islets of Langerhans of the pancreas”, as described in the instant application at page 13, lines 17-21; page 29, lines 17-24; and Examples 2 and 3.

The data presented in Huang et al. (Exhibit A) demonstrate the treatment of diabetes mellitus by the transfer of ICCs into a patient. Exhibit A teaches that 500 ICCs were introduced to each mouse; a dosage consistent with that taught by the present specification.

Exhibit A demonstrates that, in contrast to the Examiner's assertion, the transplanted pseudo-islet like aggregates (ICCs) act like healthy islet cells and effect blood glucose levels, resulting in reversal of hyperglycemia, indicative of diabetes treatment.

Exhibit A demonstrates the successful implantation of human nestin-positive cell aggregates into mice. That is, data presented in Exhibit A demonstrate that diabetes can be treated, as indicated by a reversal of hyperglycemia, without the occurrence of transplant rejection or graft rejection, even if the patient is not the donor for the nestin-positive aggregates. Thus, in view of the teachings of the specification, in further view of this publication and the abstracts of Wu, presented in Exhibits A and B in Applicants April 4, 2003 response to the December 4, 2002 Office Action, one of skill in the art would have no reason to predict that graft or transplant rejection would occur if a human patient were transplanted with pseudo-like aggregates derived from any of the following: another human, a pig, monkey rat, etc... Thus, Applicants submit that where the methods described in Exhibit A, presented herewith, as well as Exhibits A and B presented with Applicants' April 4, 2003 response, essentially mirror the teaching of the present specification, one of skill in the art would have been able to reproduce the claimed invention as of the filing date, without undue experimentation.

Although, the studies performed in Exhibit A, presented herewith, and Exhibits A and B presented with Applicants' April 4, 2003 response were conducted in an art recognized animal model of diabetes, Applicants submit that *In re Brana* also provides that such *in vivo* animal testing is sufficient to meet the patentability requirements for an invention relating to the treatment of humans. *Id.*

In view of the above, and further in view of Applicants' April 4, 2003 response to the December 4, 2002 Office Action, Applicants submit that claims 25-35 and 67-79 meet the legal requirements for enablement and respectfully request withdrawal of the 35 U.S.C. § 112, first paragraph rejection.

CONCLUSION

Applicant submits that all claims are allowable as written and respectfully request early favorable action by the Examiner. If the Examiner believes that a telephone conversation with Applicants' attorney would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney of record.

Date: May 22, 2003

Respectfully submitted,



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Phenotypic Determination and Characterization of Nestin-Positive Precursors Derived from Human Fetal Pancreas

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SUMMARY: Demand for donations to meet the requirements of pancreas or islet transplantation has prompted the search for alternative sources of β -cell replacement therapy. Earlier studies identified nestin-positive islet-derived progenitor cells (NIPs) residing in human pancreas. In the present study, we isolated and cultured human fetal NIPs that express stem cell marker *ABCG2/BCRP1*. In confluent cultures, NIPs formed three-dimensional islet-like cell clusters (ICCs). During differentiation, NIP-derived ICCs showed numerous pancreatic lineage transcripts including *insulin*, whereas *ABCG2* and *nestin* expression fell concomitantly. In addition, ICCs displayed the ability to reverse hyperglycemia in diabetic NOD-SCID mice, as well as infiltrate and form well-differentiated structures in normal mice. These cells can be cloned repeatedly and maintained in long-term culture. Our studies are the first to show NIPs derived from human fetal pancreas, which may have significant implications for future applications in stem cell therapy of diabetes. (*Lab Invest* 2003, 83:539–547).

Type 1 diabetes is a multisystem disease resulting from autoimmune destruction of pancreatic islet β cells. The most widely used treatment for type 1 diabetes is insulin injection, but this modality does not avoid many of the most common diabetic complications. In some cases, pancreatic or islet cell transplant is effective, but widespread application is limited by a shortage of available insulin-producing tissues. β cells in a normal adult rat have a life span of approximately 50 days (Finegood et al, 1995). Maintenance of blood glucose level requires β -cell neogenesis from pancreatic stem cells. Neogenesis was induced experimentally by several methods, including partial pancreatectomy, specific growth factor, and wrapping the head of the pancreas in cellophane (Bonner-Weir et al, 1993; Otonkoski et al, 1994; Rosenberg and Vinik, 1992). The ability to stimulate the growth and differentiation remains a significant scientific and clinical interest, and pancreatic stem cells may lead to numerous clinical benefits, including stem cell therapy for type 1 diabetes.

There have been a number of reports on the “elusive pancreatic stem cell” (Arias and Bendayan, 1993; Bonner-Weir et al, 2000; Cornelius et al, 1997; Schwitzgebel et al, 2000; Zulewski et al, 2001); however, a definitive molecular marker remains obscure. Some reports hold that stem cells exist in duct structures, based on the observation of islet neogenesis from duct structures in these areas in vivo (Gmyr et al,

2000; Rosenberg, 1995). Others showed the possibility of intra-islet stem cells (Fernandes et al, 1997; Guz et al, 2001), as it is known that islet implantation has a long-term curative effect. Recently the intermediate filament protein nestin, a neural stem cell marker (Lendahl et al, 1990), was found to be expressed in pancreas (Hunziker and Stein, 2000). These nestin-positive islet-derived progenitor cells (NIPs) were isolated from rat and adult human pancreases and were found to be multipotential stem cells (Zulewski et al, 2001). Furthermore, a recent study that aimed at inducing the differentiation of ES cells into pancreatic β cells achieved the production of islet-like cells from nestin-positive precursors (Lumelsky et al, 2001). Similar stem cells that could reverse diabetes in mice were isolated from prediabetic mice and transdifferentiated hepatic stem cells (Cornelius et al, 1997; Ramiya et al, 2000; Yang et al, 2002). However, no study has been reported on human fetal NIPs. In the present report, we present in vitro and in vivo experiments with NIPs derived from human fetal pancreas. Our study suggests promising implications for theoretical and clinical applications of pancreas regeneration.

Results

In Vitro Growth and Phenotype Determination of Human Fetal NIPs

To identify the composition of ducts and primary cell clusters in human fetal pancreas, we conducted immunofluorescence studies in primary culture. Three types of adherent cells were identified: large cells immunopositive for CK19 characterize the pancreatic ductal epithelium (Fig. 1A), insulin-positive β cells are found in patches with endocrine granules on one side

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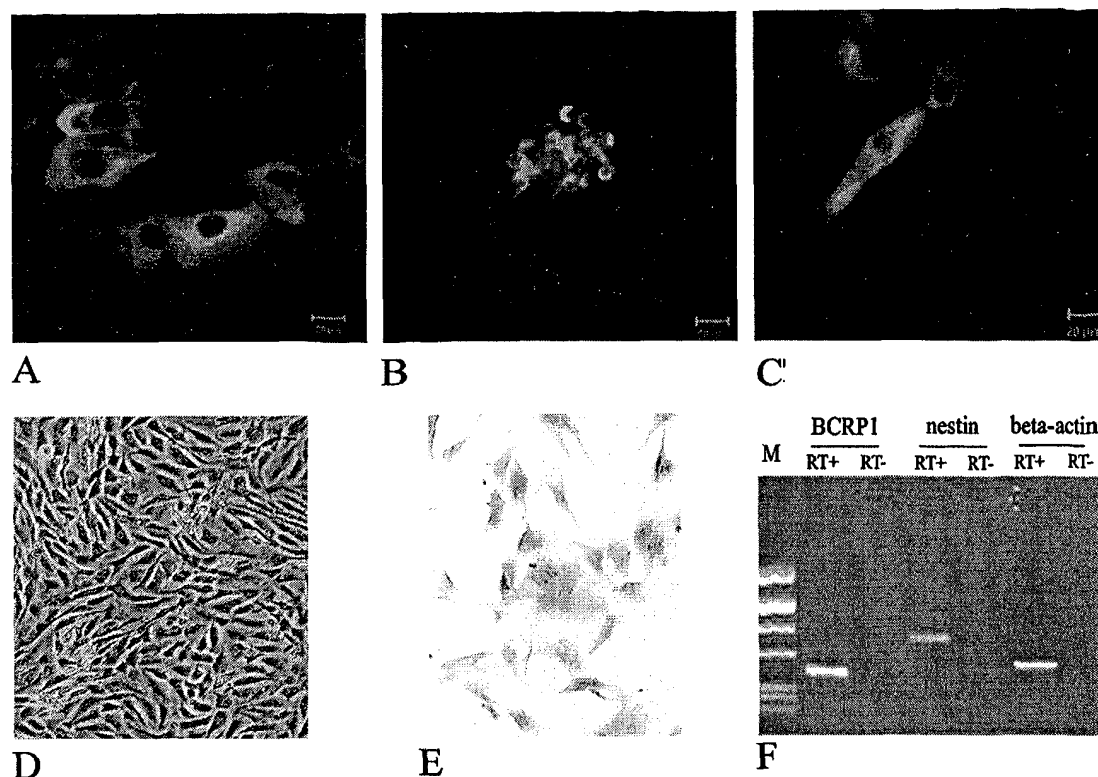


Figure 1.

A to C, Composition of adherent cells after ducts and cell clumps flattened into monolayer in primary culture. A, CK19-positive cells are epithelium like. B, Small cells in patches stained for insulin. C, Nestin-positive cells are scattered and infrequent (magnification bars = 20 μ m). D to F, Phenotypic determination of human fetal NIPs. D, Monolayer appearance of plaques of human fetal NIPs formed 2 to 3 days after selection (40 \times). E, NIPs maintained their stellate-like appearance and immunopositive for nestin (200 \times). F, RT-PCR analysis of NIP cultures showed high transcription levels of stem cell markers nestin and ABCG2.

of the cytoplasm (Fig. 1B), and stellate nestin-expressing cells are infrequent and always grow in a scattered manner (Fig. 1C). Monolayer plaques of cells formed in EHAA medium were incubated for approximately 4 weeks in the glucose-free medium with 0.5% fetal bovine serum (FBS). After this selection procedure, only a few stellate cells remained and scattered on the surface of flasks. To stimulate the growth of NIPs, we added 20 ng/ml basic fibroblast growth factor (bFGF) and 5.6 mM glucose into the culture medium. Cells underwent rapid proliferation and formed cell plaques in 2 to 3 days (Fig. 1D). Because the residual cells are so infrequent, we consider each plaque to be the progeny of a single cell. Cells in individual plaques were subcloned by removing some cells into a fresh culture dish containing identical medium. These cells maintained their stem cell-like phenotype, expressing nestin (Fig. 1E). In addition, a high level of the stem cell-specific gene *ABCG2/BCRP1* was detected by RT-PCR (Fig. 1F). Upon reaching 75% to 90% confluence, the cultures were split to maintain them in long-term culture.

In Vitro Differentiation of Human Fetal NIPs

NIPs will eventually self-assemble to form islet-like cell clusters (ICCs) if the cultures are not split. According

to development levels, ICCs will undergo three stages of differentiation.

Stage 1: NIPs Self-Assemble to Form ICCs. After NIPs reached 80% confluence, cells began to migrate and form small transparent ICCs lying on top of NIPs (Fig. 2A). This procedure typically took 10 days but is reduced to 4 to 5 days when 10% horse serum is added. We subcloned some ICCs by placing them into 24-well plates individually, allowing them to repeat the process. Cells migrated from ICCs and became confluent by 2 to 3 days. This process was repeated several times and proved to be highly reproducible. Immunohistochemistry assays revealed that newly formed ICCs were immature and composed predominantly of undifferentiated nestin-positive cells (Fig. 2B). No insulin or glucagon expression was found despite high levels of *ABCG2* and *c-MET* transcripts (Fig. 2G). Three-dimensional ICC structures formed at this stage may provide interesting evidence for further differentiation.

Stage 2: Differentiation of NIPs into Cells of Diverse Phenotypes. After subcloning, ICCs were incubated for 2 weeks in DMEM/F12 medium plus 10% horse serum and 10% FBS without bFGF. ICCs were enlarged and denser than that of stage 1 (Fig. 2C), and their number increased to 3 to 4 $\times 10^3$ per 25-cm²

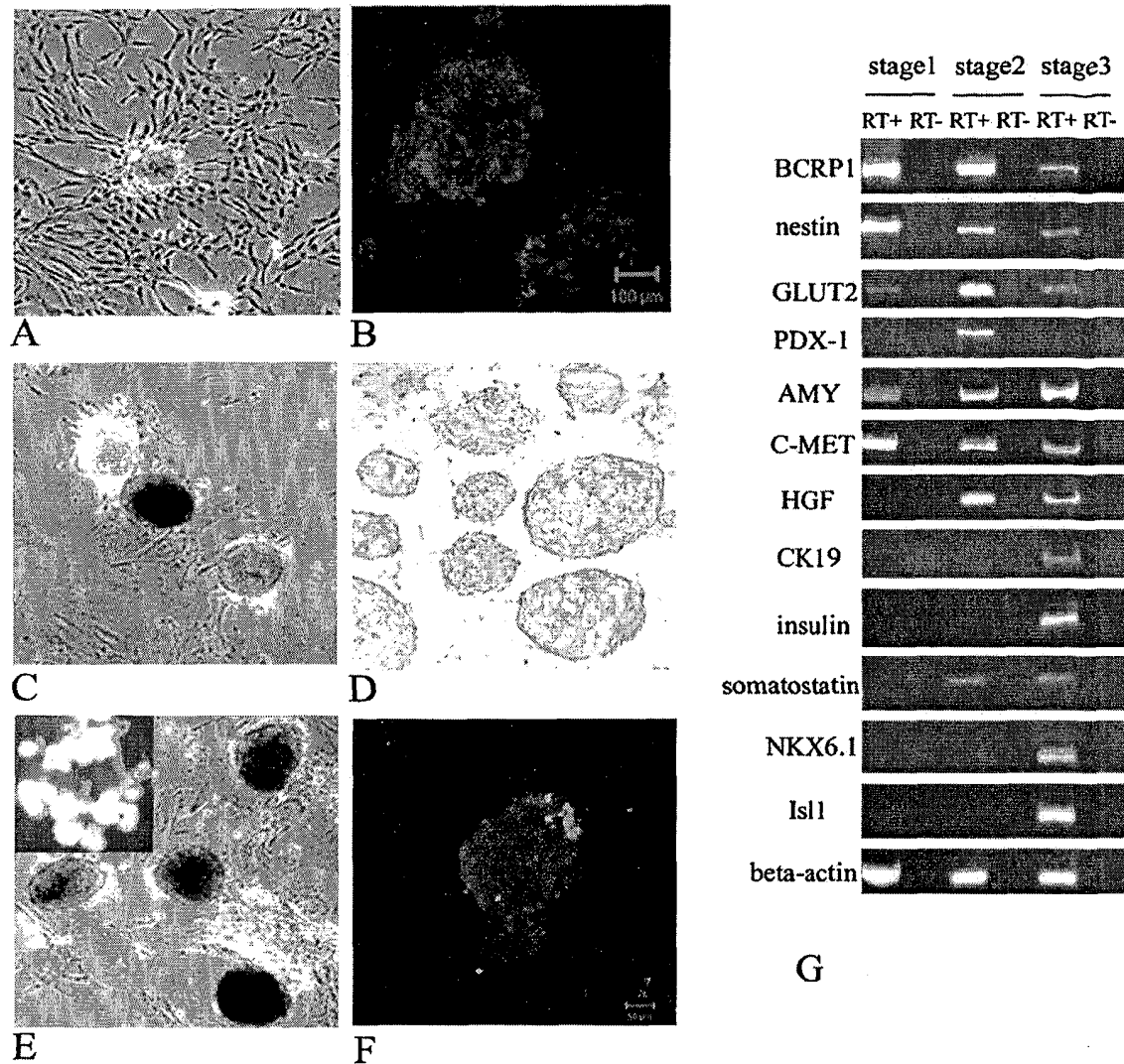


Figure 2.

Increased differentiation of NIPs in ICCs. A, Upon confluence, NIPs began to form immature ICCs (40 \times). B, ICCs of stage 1 are primarily composed of NIPs, immunostaining for nestin (bar = 100 μ m). C, After bFGF was withdrawn, ICCs of stage 2 became much bigger and more compact after 2 weeks in FBS and HS (40 \times). D, Glucagon expression in cells is close to the periphery but not in the center of ICCs of stage 2 (100 \times). E, Incubation of ICCs of stage 2 with inducers for 10 days promoted an increase in number and size of ICCs containing more specialized cells formed at stage 3 (40 \times), and many ICCs were detached to float in the medium (inset, 15 \times). F, Insulin-producing β cells were detected in ICCs of stage 3 by immunofluorescent staining (magnification bar = 50 μ m). G, RT-PCR analysis of pancreatic gene expression of ICCs, note that down-regulation of progenitor markers correlate with increasing expression of β -cell markers.

culture plate. In some cases, cells close to the periphery of ICCs expressed glucagon (Fig. 2D). There were still no insulin-positive cells in ICCs of this stage. Genes encoding the pancreatic differentiation markers *PDX-1*, *GLUT-2*, and *HGF*; endocrine markers *glucagon* and *somatostatin*; and exocrine marker *AMY* were activated in ICCs of this stage determined by RT-PCR (Fig. 2G). These results indicate that human fetal NIPs are multipotential like their adult counterpart; however, they can generate a variety of more specialized cell types.

Stage 3: Conversion of NIPs into β -Cell Phenotype. We next attempted to induce efficiently production of β cells from NIPs. A survey of inductive factors for β

cells revealed that in addition to glucose, which stimulates their maturation, the combined effect of hepatocyte growth factor (HGF), nicotinamide, insulin growth factor (IGF), and betacellulin favor differentiation of NIPs toward β cells. After ICCs of stage 2 were cultured for 10 days in DMEM/F12 (16.5 mM glucose) with above combined factors added, an increase in both the number and size of ICCs was observed with many detached and floating in the medium (Fig. 2E). Immunofluorescent staining showed that insulin-positive cells presented in ICCs (Fig. 2F), suggesting that β cells have derived from NIPs. The analysis of mRNAs implicates that expressions of β -cell markers *insulin*, *Nkx6.1*, and *Isl1* and duct cell marker *CK19*

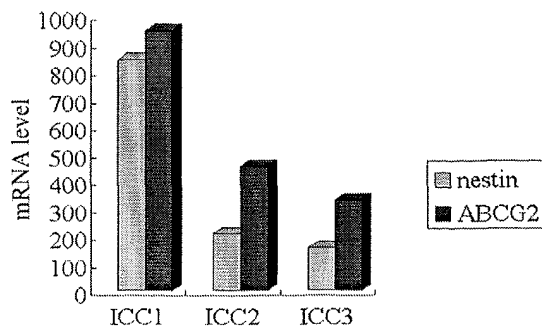


Figure 3.

Semiquantitative RT-PCR analysis of ABCG2 and nestin expression in ICCs of different stages. All values are taken from 30 cycle amplifications and are normalized to β -actin from the same sample. Note that differentiation of NIPs correlate well with the down-regulation of ABCG2 and nestin. (ICC1, ICCs of stage 1; ICC2, ICCs of stage 2; ICC3, ICCs of stage 3.)

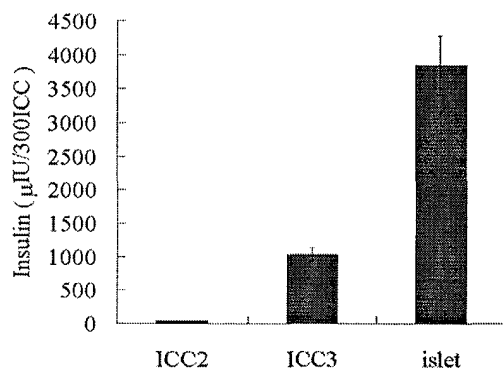


Figure 4.

Ability of NIP-derived ICCs to produce and store insulin. Note that intracellular insulin level of ICC3 is much higher than that of ICC2, while lower than that of human fetal islet ($n = 3$; ICC2, ICCs of stage 2; ICC3, ICCs of stage 3; islet, primarily cultured human islets).

present during this stage, whereas transcription level of *PDX-1* is too low to be detected (Fig. 2G). It is interesting that NIP differentiation correlates well with the down-regulation of *ABCG2* and *nestin* demon-

strated by semiquantitative RT-PCR analysis (Fig. 3). In addition, *c-MET* and *AMY* maintained high levels of expression even at the third stage of differentiation.

As showed in Figure 4, intracellular insulin content in ICCs of stage 3 is much higher than that of stage 2. However, this value of insulin level is still very low compared with that of human fetal islets, which indicate incomplete induction of NIPs. Further optimization of conditions is required to improve the efficiency of induction.

Insulin Secretion in Response to Glucose

For determining whether these insulin-positive cells could release insulin as do normal β cells, ICCs of stage 3 were challenged by glucose as well as other secretagogues. As a result, glucose-challenged ICCs secreted a certain amount of insulin and this glucose-responsive insulin secretion seems to take a dose-dependent manner like primary pancreatic islets do in vitro (Fig. 5A). Furthermore, all of the secretagogues tested also induced insulin secretion in the presence of 5 mM glucose (Fig. 5B), which means that ICCs use normal pancreatic mechanisms to regulate insulin release.

Preliminary In Vivo Studies of Human Fetal NIPs

To investigate the ability of human fetal NIPs to reverse hyperglycemia, we implanted 500 ICCs into the subcapsular region of the left kidney of streptozotocin (STZ)-induced diabetic mice, and blood glucose levels were monitored at regular intervals. Four days after implantation, diabetic mice showed decreases in blood glucose (Fig. 6A). Seven days later, the average blood glucose level of the eight mice approached normal levels and was stable up to 40 days. In contrast, mice of the STZ group that did not receive ICCs showed persistent hyperglycemia. Statistical assay showed that implantation of ICCs achieved a significant decrease in blood glucose ($p < 0.05$). Histologic and immunohistochemical examinations revealed neovascularization and insulin-positive clusters in implanted sites (data not showed). These results

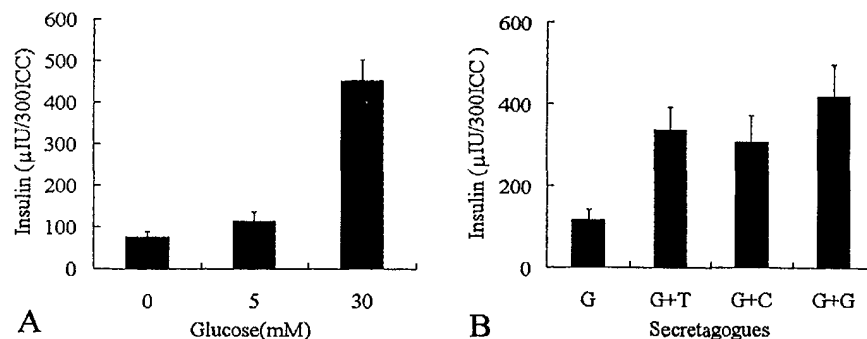


Figure 5.

ICCs of stage 3 release insulin in response to glucose and secretagogues. A, ICCs secrete insulin after glucose challenge, indicating functional β cells in ICCs. B, Insulin release in response to various secretagogues in the presence of 5 mM glucose ($n = 3$; G, 5 mM glucose; G+T, 5 mM glucose + 1 mM tolbutamide; G+C, 5 mM glucose + 1 mM carbachol; G+G, 5 mM glucose + 1 mM GLP-1).

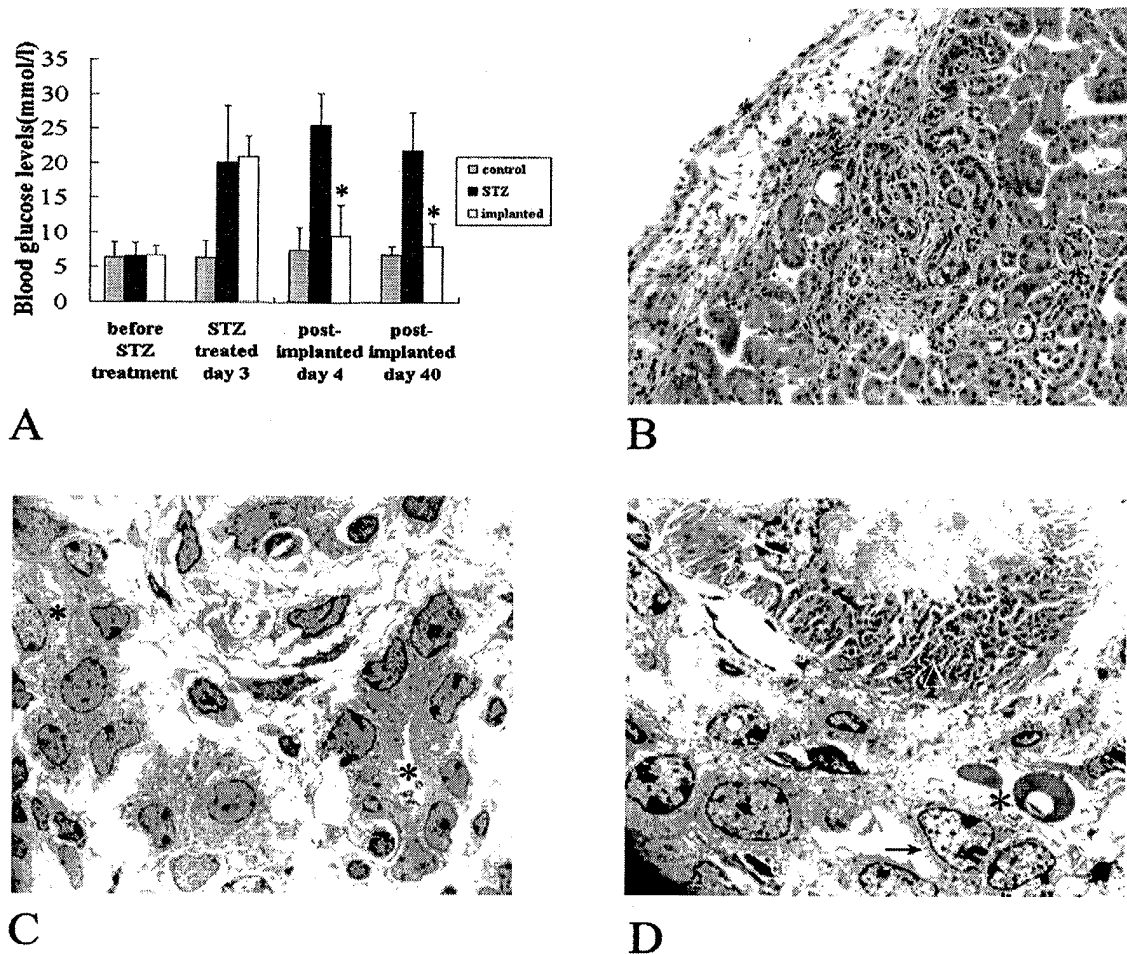


Figure 6.

In vivo phenotypic analysis of NIPs. A, Reversal of hyperglycemia in diabetic mice ($n = 8$). All NOD-Scid mice became hyperglycemic 3 days after STZ treatment. Blood glucose levels of implanted group approached normal 4 days after 500 ICCs were implanted and were kept stable up to 40 days, whereas the STZ group showed persistent hyperglycemia. Normal mice without any treatment were selected as control. * $p < 0.05$ denotes a significant difference when compared with values of STZ group on the same day. B to D, ICCs implanted in normal NOD-Scid mice overproliferated and infiltrated into renal parenchyma. B, Histologic sections of kidney stained with H&E showed that NIPs formed various structures and infiltrated from implant site to renal parenchyma (200 \times). C, Ultrastructural study of subcapsular region showed cell clusters and duct structures (*) (1600 \times). D, Renal parenchymal ultrastructure showed that duct-like structures with microvilli (→) formed adjacent to renal tubule (^) and blood vessel (*) (2100 \times).

provide promising evidence that ICCs of stage 3 produce adequate insulin in vivo and function in reducing the blood glucose level of diabetic mice.

As control, ICCs of stage 3 were implanted into three normal NOD-Scid mice. Kidneys were removed 40 days after implantation. Histologic sections of implant sites showed that ICCs coalesced to form a large mass that infiltrates the renal parenchyma and forms various structures that seem to be ducts, small compact cell clusters, blood vessels, and scattered cells (Fig. 6B). Ultrastructural studies of the subcapsular region (Fig. 6C) and renal parenchyma further demonstrated this infiltration (Fig. 6D), and such phenomena were found in all implant sites of normal mice.

Discussion

The present study provides evidence that NIPs derived from human fetal pancreas are multipotential and

have the capacity to differentiate into functional β cells. This is consistent with adult NIPs (Abraham et al, 2002; Ramiya et al, 2000; Zulewski et al, 2001) and ES-derived nestin-positive cells (Lumelsky et al, 2001). Nestin was initially found in neural stem cells (Lendahl et al, 1990) and later in myoblast and pancreatic precursors and (Hunziker and Stein, 2000; Wroblewski et al, 1997). Nestin-positive cells harbored in pancreatic ducts and islets are similar to other multipotential stem cells both in proliferation behavior and on a molecular level, and that their number increases greatly in regenerating adult pancreas suggests a contribution in the process of pancreas regeneration (Lardon et al, 2002). In addition, it was reported that nestin-positive cells in pancreas were found in the stroma of pancreatic ducts and common in connective tissue, as well as in the islets (Selander and Edlund, 2002). These cells were also found in endothelial cells

from neocapillaries and in hepatic stellate cells (Niki et al, 1999). Additional studies are required to determine conclusively whether heterogeneity in the population of nestin-positive cells is the cause of the current controversy and whether NIPs generate insulin-producing cells by trans-differentiation.

During the process of expansion and differentiation, the culture conditions were changed several times. Relatively low concentrations of serum and bFGF were applied for expansion of NIPs, as low concentration of serum is essential for preventing neural stem cells from differentiation (Gage, 2000; Okabe et al, 1996). In addition, bFGF is mitogenic for neural stem cells (Vescovi et al, 1993), and attenuation of bFGF signaling pathway might cause diabetes (Hart et al, 2000). Besides removal of bFGF, induction of differentiation of NIPs depends mainly on two factors: a concomitant increase in the concentration of glucose, a main regulator for the maturation of β cells (Melloul and Cerasi, 1994; Rosenberg and Vinik, 1992), and the particular niche in three-dimensional ICC structures. The microenvironment, known as a "niche," is essential for certain stem cells to proliferate and differentiate. It is believed that niche is more important than cell lineage history (Spradling et al, 2001). Niches control stem cells by signals and interactions between cells. Three-dimensional structure made up by extra cellular matrigel is an in vitro niche in which islets are formed from ducts (Bonner-Weir et al, 2000). In our study, the three-dimensional ICC structures formed during NIP differentiation resemble morphologically those described by other researchers (Bonner-Weir et al, 2000; Ramiya et al, 2000; Yang et al, 2002; Zulewski et al, 2001). ICCs may provide cell-cell interactions and an appropriate niche for the differentiation of human fetal NIPs. We encountered the problem of incomplete induction, which is a common difficulty in studies of stem cells. Even at end stage of differentiation, some undifferentiated cells and other cell types remain. Additional efforts to optimize conditions and enhance the efficiency of induction are under way.

In our culture of NIPs, genes encoding pancreatic proteins are activated sequentially. In their undifferentiated stage, NIPs are nestin and ABCG2 positive but PDX-1 and insulin negative. Our detection of ABCG2 expression in human fetal pancreatic NIPs is in agreement with a recent report that indicates side population cells in adult pancreas (Lechner et al, 2002). ABCG2 expression had also been previously detected in ES cells, hematopoietic stem cells, muscle stem cells, marrow stem cells (Geschwind et al, 2001; Zhou et al, 2001), neural stem cells (Geschwind et al, 2001), and premature hematopoietic progenitor cells (Scharenberg et al, 2002). It has been speculated that ABCG2 may pump differentiation factors out of the cell to keep the cell in an undifferentiated state (Scharenberg et al, 2002). When NIPs begin to differentiate and form ICCs, tissue-specific genes expressed at this stage include pancreatic markers such as PDX-1, GLUT-2, glucagon, and AMY. Transcription of insulin is undetectable in ICCs until further induced. It is interesting that PDX-1 expression falls at stage 3,

and this drop may be predictable as it was previously demonstrated that transcript of PDX-1 is undetectable in the islets of adult rodents (Stoffers et al, 1999). Similar phenomena were also observed in ES-derived insulin-secreting cells (Lumelsky et al, 2001) and transdifferentiated adult hepatic stem cells (Yang et al, 2002). Despite this down-regulation of PDX-1, which may be due to posttranscriptional control (Stoffers et al, 1999), β -cell phenotype was reconfirmed by detecting transcripts of various β -cell markers such as insulin, Nkx6.1, and Isl1. In addition, c-MET maintained high levels throughout differentiation, and because c-MET is closely related to cell mobility, it may contribute to aggregation of NIPs and formation of ICCs. Semiquantitative RT-PCR showed that differentiation of NIPs correlate well with the down-regulation of ABCG2 and nestin, suggesting that nestin may have important roles in keeping stem cells from differentiation as ABCG2 does. The coexpression of nestin and ABCG2 demonstrated herein warrants additional studies and might indicate a broader role for nestin as a general marker of stem/progenitor cells.

Stem cells have offered much hope, and stem cell therapies in animal experiments have been proved as an effective alternative. As human disease treatment must be both safe and effective, safety should be considered as an issue of priority. One safety issue is that tumors may originate from the transformation of stem cells. Similar signaling pathways may also regulate self-renewal in stem cells and cancer cells, and the latter may include "cancer stem cells" (Reya et al, 2001). ABCG2, a member of ABC (ATP binding cassette transporter) superfamily, not only is responsible for multidrug resistance in many cancer cell lines (Ross et al, 1999) but also is found to help maintain the unique properties of stem cells (Zhou et al, 2001). It is possible that ABCG2 is one of the molecules that may implicate stem cells with cancer. When we implanted stage 3 ICCs into normal NOD-Scid mice, they overproliferated and infiltrated into renal parenchyma. Tumor-like structures were observed, but the expansion velocity is slower than tumor cells. It is notable that no infiltrating cells were found in diabetic mice that received with stage 3 ICCs, possibly because of stimulated differentiation of NIPs under the specific environment in diabetic mice. The most important factor may be glucagon-like peptide-1 (GLP-1), which was found with a robust increase in STZ-induced diabetic rats and may play a role in neogenesis of β cells (Nie et al, 2000). Recently, GLP-1 was used successfully to induce differentiation of NIPs into insulin-producing cells (Abraham et al, 2002). Other factors may include concentrated blood glucose, a main regulator during maturation of pancreatic β cells (Leibowitz et al, 2001), and serum from diabetic mice, which was used as the main inductive factor in in vitro cultured pancreatic stem cells (Cornelius et al, 1997). It is widely known that ES cells are capable of forming tumors in vivo; similar tumor-forming ability has not been observed in adult stem cells until now. Thus, additional research is needed to determine whether the overproliferation found in our study is malignant.

Materials and Methods

Isolation, Culture, and Induction of NIPs

Twelve- to 21-week-old human fetal pancreata were collected at the Obstetrical and Gynecological Hospital of Fudan University. Gestational age was determined by several previously described criteria (Mercer et al, 1987; Streeter, 1920). Freshly obtained pancreata were washed three times in PBS, extraneous material was carefully dissected away, and pancreata were minced gently into smaller fragments. After a single wash in HBSS containing 10 mM HEPES to remove blood cells, the fragments were subjected to 5.5 mg/ml collagenase IV in a shaking water bath (37° C). Duct and primary cell clusters were confirmed by inverted microscopy. The collagenase was then purged by centrifugation, followed by a single wash in cold HBSS. Duct and primary cell clusters were subsequently handpicked and placed into a tissue culture flask containing EHAA medium supplemented with 2% FBS. After a 48-hour incubation at 37° C with 5% CO₂, most cells attached to the bottom and slightly spread out. For selecting nestin-positive cells, adherent cells were further cultured in DMEM (without glucose) supplemented with 0.5% FBS and were incubated undisturbed for 3 to 4 weeks until the majority of the original cells died. The remaining live cells were then half refreshed by DMEM/F12 (11.1 mM glucose) supplemented with 10% FBS and 20 ng/ml bFGF to favor expansion. Cells proliferated rapidly and were maintained for approximately 1 year by serial splits before reaching confluence. For inducing differentiation, NIPs were cultured in DMEM/F12 containing high glucose (16.5 mM) and cocktail of several growth factors, including 10 ng/ml IGF, 500 pmol/L betacellulin, 10 ng/ml HGF, and 10 mM nicotinamide.

Immunohistochemistry

ICCs of various stages were fixed for 2 hours in 4% paraformaldehyde and embedded in OCT. Cryosections were stained by the ABC method according to standard protocols. The following antibodies were used after dilution: mouse anti-human nestin antibody (1:100; Chemicon), monoclonal anti-insulin (1:1000; Sigma), monoclonal anti-glucagon (1:2000; Sigma), monoclonal anti-cytokeratin peptide 19 (1:50; Sigma), FITC-conjugated affinipure donkey anti-mouse IgG(H+L) (1:200; Jackson), and biotin-Sp-conjugated affinipure goat anti-mouse IgG(H+L) (1:500; Jackson). Detection was accomplished using Streptavidin/Streptavidine HRP (1:200; Dako). The reaction substrate was DAB (Sangon). BSA was used in place of primary antibodies on control slides. Counterstaining was carried out with hematoxylin, and fluorescent images were obtained using a Zeiss LSM-510 laser confocal microscope.

Measurement of Intracellular and Secreted Insulin by Immunoenzymatic Assay

For determining total cellular insulin content, insulin was extracted from ICCs and equal amount of primar-

ily cultured human fetal islets. Cells were treated with lysis buffer, followed by cell sonication. For insulin secretion assay, 300 ICCs of stage 3 were incubated in medium for 1 week, washed twice with serum-free medium, and then stimulated for 2 hours in serum-free medium containing glucose of various concentrations. In addition, ICCs were challenged by 5 mM glucose in conjunction with 1 mM tolbutamide, 1 mM carbachol, and 1 mM glucagon-like peptide-1, respectively. Supernatants were collected for the insulin secretion detection. Insulin concentrations were measured by magnetic solid-phase enzyme immunoassay (Insulin Serozyme kit; Bio-Ekon Diagnostics Products). The reagent is highly specific for the detection of human insulin, and cross-reaction to proinsulin or c-peptide is < 0.01%.

RT-PCR Assay

Total RNA was prepared from cultured NIPs and ICCs using Trizol (GIBCO). After purification and reverse transcription, the cDNA was amplified by PCR for 30 cycles. Primer sets were designed on the basis of published reports or GenBank and synthesized by Sangon Company. Forward and reverse primer sequences from 5' to 3' direction and the lengths of the amplified products were as follows: *PDX-1*, GGATGAAGTCTACCAAAGCTCACGC, CCAGATCTTGATGTGTCTCTCGGTC (218 bp); *Amylase*, GCTGGGCTCAGTATTCCTCCAAATAC, GACGACAATCTCTGA CCTGAGTAGC (492 bp); *β-actin*, TGGCACCACACCTTCTCAATGAGC, GCACAGCTTCTCCTTAATGTACGC (396 bp); *CK19*, AGGAAATCATCTCAGGAGGAAGGGC, AAAGCAGATCTTCGGGAGCTACC (783 bp); *somatostatin*, GTTCTGCAGAAAGTCTCGG, AGTCTTGCAGCCAGCTTTG (223 bp); *nestin*, AGAGGGGAATTCCTGGAG, CTGAGGACCAGGACTCTCTA (495 bp); *HGF*, GCATCAAATGTCAGCCCTGGG, CAACGCTGACATGGAATTC (534 bp); *c-MET*, CAATGTGAGATGTCTCCAGC, CCTGTGATGTCAGGCAGA (559 bp); *GLUT2*, GCAGCTGCTCAACTAATCAC, TCAGCAGCACAAGTCCCACT (909 bp); *insulin*, TCACACCTGGTGGAAGCTC, ACAATGCCACGCTTCTGC (179 bp); *Nkx6.1*, GATCCATTTTGTTGGA-CAAAGA, CACGGCCAAGAAGAGGCAGGACT (221 bp); *Isl1*, CTAAATTGGACTCCTAGAT, GGATTGGAATGCGATCGCG (280 bp); *ABCG2/BCRP1*, GGCCTCAGGAAGACTTATGT, AAGGAGGTGGTGTAGCTGT (342 bp). The annealing temperature was 50° C for *PDX-1* and *insulin*; 51.5° C for *CK19*, *Isl1*, and *GLUT2*; 52.5° C for *somatostatin* and *NKX6.1*; 53.5° C for *AMY* and *nestin*; 56.5° C for *HGF* and *ABCG2/BCRP1*; 57.5° C for *actin*; and 60° C for *c-MET*. Agarose gel (1.7%) was used to separate the PCR products.

Semiquantitative RT-PCR assay of both *ABCG2* and *nestin* transcripts was performed by amplification of the *β-actin* normalized RT reactions along with quantitative standards. Amplified product values were normalized to *β-actin* for intersample comparisons or to standard curves for intergene comparisons within a given sample.

Transplantation Studies

NOD-Scid mice obtained from Jackson Laboratory were bred and maintained in the IVC (individual cage) mouse facility of the Department of Laboratory Animal Science of Shanghai Second Medical University. Eight- to 12-week-old male mice were selected for allogeneic transplantations. All animal studies were in accordance with Shanghai Second Medical University institutional guidelines. Sixteen mice were divided into two groups: (a) the group that received implant and (b) the STZ group. Experimental diabetes was induced by a single intraperitoneal injection of STZ as previously described (Lumelsky et al, 2001). Briefly, STZ (Sigma) was freshly dissolved in 0.1 M of citrate buffer (pH 7.4) and injected into NOD-Scid mice at a dose of 150 mg/kg body weight. Blood glucose level was determined with a blood glucose monitor and lancing device (Roche, GLUCOTREND soft test system). After a stable level of hyperglycemia was reached (typically 48 to 72 hours), animals were prepared to receive implants. Eight NOD-Scid mice were anesthetized by injecting 600 μ l of 2.5% pentobarbital sodium. The left kidney was exposed through a small incision in the flank, and approximately 500 ICCs of stage 3 were implanted into the subcapsular region via a small glass catheter, and the incision was closed by suture. STZ-treated NOD-Scid mice received an implant of physiologic saline as a control. Eight normal NOD-Scid mice did not receive any treatment as control. For investigating the in vivo potential of NIPs, an equal amount of stage 3 ICCs were implanted into normal NOD-Scid mice. All animals were killed 40 days after the procedure, and the left kidneys were excised and were fixed in 4% paraformaldehyde and 2% glutaraldehyde for further research.

Ultrastructural Analysis

Kidneys of normal NOD-Scid mice were excised 40 days after ICCs were implanted, and were fixed with 2% glutaraldehyde, postfixed with 1% OsO₄, dehydrated in graded acetone solutions, and embedded in epoxy resin 618. Ultrathin sections were cut with a Leica Ultracut R and counterstained with uranium acetate and lead citrate. Ultrastructural study was performed using a Philip CM120 TEM.

Statistical Analysis

Results are expressed as means \pm SEM. Statistical analysis of the data was performed using Student's *t* test. *p* < 0.05 was considered to be significant. Statistics software used is SAS 6.2.

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